

# Mutagenic Activity of Phthalate Esters in Bacterial Liquid Suspension Assays

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The mutagenic activities of several phthalate esters have been evaluated in an 8-azaguanine resistance assay in *Salmonella typhimurium*. Three phthalate esters were found to be mutagenic: dimethyl phthalate, diethyl phthalate and di-*n*-butyl phthalate. A number of other phthalate esters were not found to be mutagenic, including di(2-ethylhexyl) phthalate, di-*n*-octyl phthalate, diallyl phthalate, diisobutyl phthalate and diisodecyl phthalate. A metabolite of di(2-ethylhexyl) phthalate, 2-ethylhexanol, was also noted to be mutagenic. The mutagenic activity of this agent and others in this series was dose dependent but weak. No dose-response curve exceeded more than 3.5 times background at maximally testable concentrations. A liquid suspension histidine reversion assay of dimethyl phthalate showed levels of mutagenic activity similar to that observed in the azaguanine resistance assay. The data suggest a need for further investigation of the mutagenic potential of these agents in other assay systems.

## Introduction

The phthalate esters are widely used in the manufacture of plastics and are also used for a variety of other commercial purposes. The uses and biological effects of phthalate esters have been described in detail by others in this volume and in previous publications (1). Among the adverse biological effects of phthalate esters is an embryotoxic effect reported by Singh et al. (2). This increased embryotoxic effect is suggestive of possible mutagenic and carcinogenic activity. In this paper we report on the mutagenic activity of several phthalate esters in 8-azaguanine resistance assays in *S. typhimurium*.

## Materials and Methods

### Bacteria

*Salmonella typhimurium* strain TA100 was obtained from Dr. Philip E. Hartman of the Johns Hopkins University.

### Phthalate Esters

Ten phthalate esters and analogs were obtained from the NTP repository and were tested for mutagenic activity without further purification. The compounds tested and the commercial sources were, dimethyl phthalate (Aldrich), diethyl phthalate (MRI), di-*n*-butyl phthalate (Aldrich), diisobutyl phthalate (Eastman), diallyl phthalate (Litton Bionetics), di-*n*-octyl phthalate (Kodak), di(2-ethylhexyl) phthalate (MRI), di(2-ethylhexyl) adipate (Pfaltz and Bauer), diisodecyl phthalate (Pfaltz and Bauer) and 2-ethylhexanol (Aldrich). All compounds were dissolved in DMSO (Aldrich, Gold Label) prior to being added to the assay system.

### Assays

Bacterial mutagenicity assays were conducted in liquid suspension by using a modification of previously described techniques (3, 4). The bacteria were tested for mutation to azaguanine resistance as well as reversion to histidine prototrophy. In azaguanine resistance assays,  $10^6$  *Salmonella* organisms were inoculated into a broth containing 2% brain-heart infusion (BHI), 0.1 mg/ml histidine, 0.01 mg/ml biotin and 2% glucose in Vogel-Bonner medium and

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were incubated 16 hr at 37°C with shaking. The cells were subsequently centrifuged at 1000 *g* for 20 min, resuspended in an equal volume of Vogel-Bonner medium and cooled to 0°C before use. A 50  $\mu$ l portion of this bacterial suspension (containing  $2.4 \times 10^9$  cells/ $\mu$ l) and 25 ml of test chemical dissolved in DMSO were added to 1 ml of Ca-Mg free phosphate-buffered saline (PBS) pH 7.0. In experiments with S9, 10  $\mu$ l of S9 and 10  $\mu$ l of cofactors were also added to the incubation medium. The S9 and the cofactors were prepared as previously described (5). The bacteria were exposed to the test chemical for 1 hr in PBS, followed by 3 hr in Vogel-Bonner medium supplemented with 10% BHI and fresh mutagen. In experiments with S9, fresh S9 and cofactors were added as described above. Following exposure to the mutagen, the cells were centrifuged at 1000 *g* for 15 min and resuspended in PBS, pH 7.0. The bacteria were diluted 1:25 and plated on 1.2% Vogel-Bonner agar containing 8-azaguanine and excess citrate as described previously (3). The cells were diluted by an additional factor of  $2 \times 10^4$  and plated on Vogel-Bonner agar without azaguanine.

Histidine reversion assays were conducted essentially as described above with the following modifications. Assay volume was expanded to 5 ml with corresponding increases in the amounts of bacteria and mutagen. Upon termination of the exposure, the cells were centrifuged twice to remove any excess histidine and were resuspended in a volume of 1 ml PBS. Plating of *Salmonella* (250  $\mu$ l/plate) was directly on Vogel-Bonner agar containing no histidine. The number of cells plated was determined by diluting  $10^6$  and plating on agar with excess histidine.

Statistical significance was determined by analysis of variance of log transformed data.

## Results

The results of our studies are summarized in Table 1. Only three of the phthalate esters tested were mutagenic: dimethyl phthalate, diethyl phthalate and di-*n*-butyl phthalate. The mutagenic response of these agents in the azaguanine resistance assay is illustrated in Figures 1-3. All of the phthalate esters tested were noted to be weak mutagens. The maximum observed increase in mutant frequency was only 3.5 times background for dimethyl phthalate and 2-ethylhexanol (Fig. 4) and was less for other compounds tested. The maximal concentration tested in these studies was determined by either the limits of solubility in aqueous solution or cytotoxicity exceeding more than 90% of control values. The concentrations required to produce a mutagenic effect ranged from 5 to 10 *mM* dimethyl phthalate to 0.09–0.18 *mM* di-*n*-butyl phthalate. In addition to

the weak mutagenic response, the phthalate esters were also noted to be highly cytotoxic at concentrations which were mutagenic. Under the conditions of the assay, absolute increases in mutant frequency were not observed at any concentration tested. However, when increased numbers of cells were plated in order to maintain a concentration of  $4 \times 10^6$  viable cells on the selective plate, absolute increases in mutant number were observed which were consistent with the observed increase in mutant frequency seen in Figures 1-3. These observations are consistent with previous observations (J. Seed, unpublished) that mutant yield in this assay is independent of cell density (3). The presence of S9 was not important for the detection of mutagenic activity. In fact, S9 inhibited the mutagenic response of the phthalate esters in this assay. Increasing the concentration of S9 fivefold completely blocked the mutagenic response of dimethyl phthalate. The mutagenic response to the phthalate esters was not limited to azaguanine resistance assays. Studies on the mutagenic activity of dimethyl phthalate in liquid suspension histidine reversion assays also demonstrated a dose-related increase in revertant frequency between 5 and 10 *mM* dimethyl phthalate (Fig. 5).

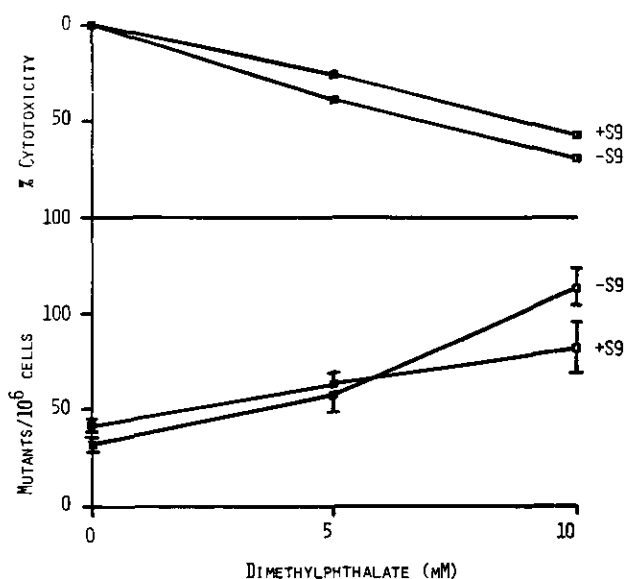


FIGURE 1. Mutagenic response of *Salmonella* to dimethyl phthalate in 8-azaguanine resistance assay. Data represent the means of four independent experiments. Response was statistically different from control as determined by analysis of variance of transformed data ( $F_{3,8} = 37.3$ ;  $p < 0.001$ ).

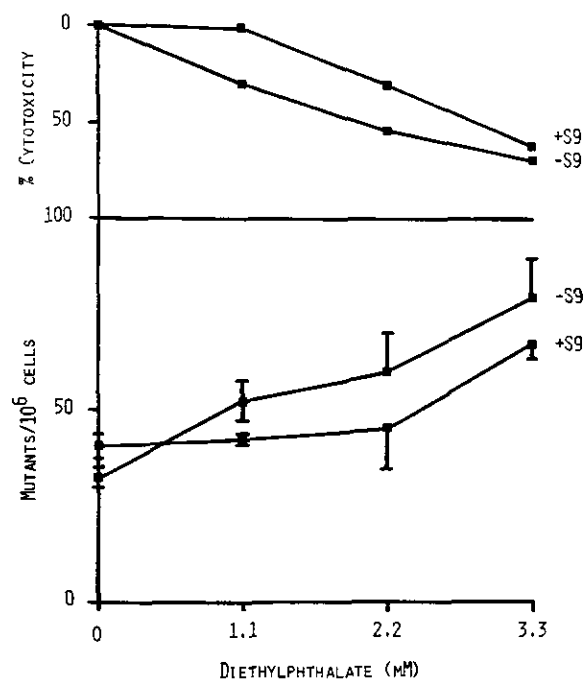


FIGURE 2. Mutagenic response of *Salmonella* to diethyl phthalate in 8-azaguanine resistance assay. Data represent the means of three independent experiments. Response was statistically different from control as determined by analysis of variance of transformed data ( $F_{2,9} = 13.7$ ;  $p < 0.005$ ).

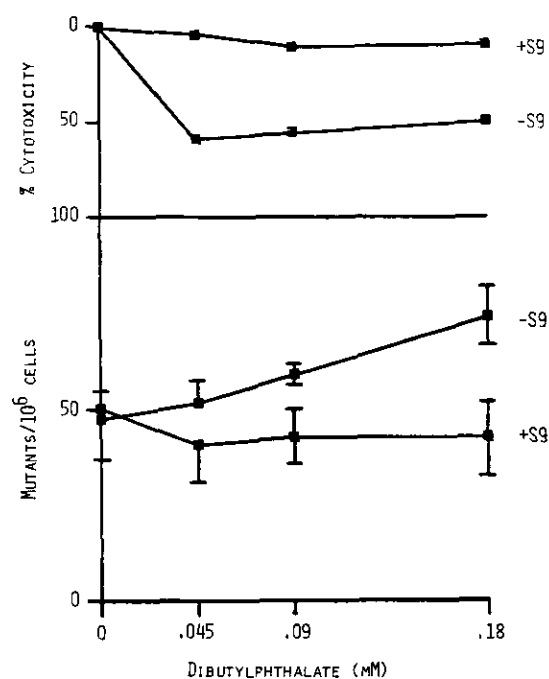


FIGURE 3. Mutagenic response of *Salmonella* to dibutyl phthalate in 8-azaguanine resistance assay. Data represent the means of three independent experiments. Response was statistically different from control as determined by analysis of variance of transformed data ( $F_{2,9} = 6.38$ ;  $p < 0.025$ ).

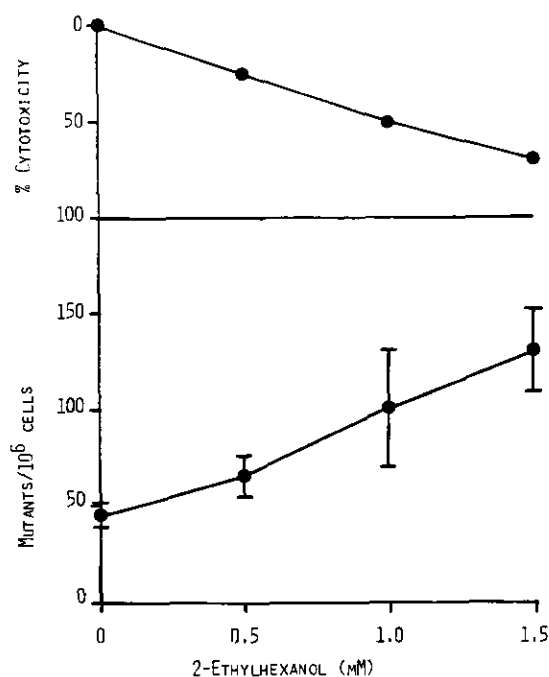


FIGURE 4. Mutagenic response of *Salmonella* to 2-ethylhexanol in 8-azaguanine resistance assay. Data represent the means of three independent experiments.

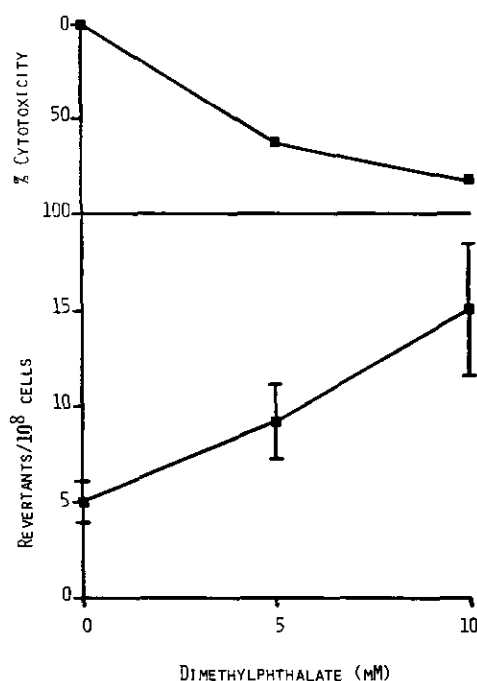


FIGURE 5. Mutagenic response of *Salmonella* to dimethyl phthalate in histidine reversion assay. Data represent the means of three independent experiments.

**Table 1. Phthalate esters and related compounds tested for mutagenic activity in azaguanine resistance assays with *Salmonella typhimurium*.**

Compound	Assay result
Dimethyl phthalate	+
Diethyl phthalate	+
Diallyl phthalate	-
Dibutyl phthalate	+
Di- <i>n</i> -octyl phthalate	-
Di(2-ethylhexyl) phthalate	-
Di(2-ethylhexyl) adipate	-
2-Ethylhexanol	+
Diisodecyl phthalate	-
Diisobutyl phthalate	-

## Discussion

The results of these studies indicate that there may be some mutagenic activity associated with certain phthalate esters. The mutagenic activity which has been observed is, however, very weak. Experiments in our laboratories have failed to detect significant mutagenic activity associated with the phthalate esters in the Ames agar plate assay although others have reported an increase using certain modifications of this test (6). Such an observation may not be surprising in view of the generally low levels of mutagenic activity observed. Liquid suspension assays are frequently more sensitive than plate assays (4, 7-10), the increase in sensitivity depending on the compound being tested. In the case of compounds such as the phthalate esters, where significant levels of cytotoxicity are associated with a weak mutagenic response, assays such as the one employed in the current study may be the most reliable indicators of a mutagenic effect.

The fact that certain of the phthalate esters are weak mutagens does not necessarily imply that they are weak carcinogens. It is difficult if not impossible to draw quantitative conclusions on the basis of a bacterial assay alone. In fact, when one is working with such low levels of mutagenic activity

as are reported in this paper, it is not certain whether or not there is even a reasonable qualitative correlation between mutagenicity and carcinogenicity. Further studies are required both *in vivo* and *in vitro* before the mutagenic potential of these compounds can be adequately evaluated.

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